Interaction of the N-Methyl-D-Aspartic Acid Receptor NR2D Subunit with the c-Abl Tyrosine Kinase*

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Robert T. Glover‡, Maria Angiolieri‡, Steven Kelly‡, Daniel T. Monaghan‡, Jean Y. J. Wang**, Thomas E. Smithgall‡, and Amy L. Buller‡§$§

From the ‡Department of Neuroscience, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, the Department of Biology, University of Nebraska, Omaha, Nebraska 68154, the §Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, the ¶Department of Pharmacology, University of Nebraska Medical Center, Omaha, Nebraska 68198, and the **Department of Biology, University of California San Diego, La Jolla, California 92039

The COOH-terminal domain of the NR2D subunit of the NMDA receptor contains proline-rich regions that show striking homology to sequences known to bind to Src homology 3 (SH3) domains. To determine whether the proline-rich region of the NR2D subunit interacts with specific SH3 domains, in vitro SH3 domain binding assays were performed. A proline-rich fragment of the NR2D subunit (2D666–1064) bound to the Abi SH3 domain but not to the SH3 domains from Src, Fyn, Grb2, GAP, or phospholipase C-γ (PLC-γ). Co-immunoprecipitation of NR2D with Abl suggests stable association of NR2D and Abl in transfected cells. The SH3 domain plays an important role in the negative regulation of Abl kinase activity. To determine whether the interaction of NR2D with the Abi SH3 domain alters Abl kinase activity, Abl was expressed alone or with NR2D in 293T cells. Auto-phosphorylation of Abl was readily observed when Abl was expressed alone. However, co-expression of Abl with 2D666–1064 or full-length NR2D inhibited autophosphorylation. 2D666–1064 did not inhibit ΔSH3 Abl, indicating a requirement for the Abl SH3 domain in the inhibitory effect. Similarly, 2D666–1064 did not inhibit the catalytic activity of Abl-PP, which contains two point mutations in the SH2-kinase linker domain that release the negative kinase regulation by the SH3 domain. In contrast, the full-length NR2D subunit partially inhibited the autokinase activity of both ΔSH3 Abi and Abi-PP, suggesting that NR2D and Abl may interact at multiple sites. Taken together, the data in this report provide the first evidence for a novel inhibitory interaction between the NR2D subunit of the NMDA receptor and the Abi tyrosine kinase.

NMDA1 receptors mediate synaptic transmission and neural plasticity at many sites in the mammalian CNS (1, 2). NMDA receptors are also involved in epileptiform activity and neuronal cell death in a number of experimental and pathological conditions (3). Two NMDA receptor subunit families (NR1a-h and NR2A-D) have been identified; alternative splicing of a single NR1 gene generates eight isoforms with distinct functional properties, while heterogeneity within the NR2 subunit family results from expression of four closely related genes (for review see, Refs. 4 and 5). Native NMDA receptors are believed to be oligomeric complexes formed from combinations of NR1 and NR2 subunits, with the NR2 subunit imparting distinct functional and pharmacological properties (6–10). The four NR2 subunits show distinct patterns of expression in brain that parallel the distribution of native receptor subtypes (10). NR2D subunit expression is developmentally regulated, with widespread distribution in embryonic and neonatal diencephalon, brainstem, and spinal cord (11, 12) and lower levels of expression in adult brain (10, 13).

Recent evidence has demonstrated that the NMDA receptor interacts with a number of cellular signaling proteins that may modulate the structure, function, and localization of the receptor (14–18). Intracellular signaling proteins, including some nonreceptor protein-tyrosine kinases, frequently contain modular domains that mediate interactions with effector and regulatory proteins. Src homology 3 (SH3) domains mediate protein-protein interactions with proline-rich target sequences that adopt a left-handed helical conformation (polyproline type II helix (19, 20)). In the case of the tyrosine kinases Src and Abi, SH3 domains function in both negative regulation of kinase activity and substrate recruitment (21–24). SH3 domains are also found in many other proteins involved in signal transduction and may be involved in cytoskeletal organization and the targeting of signaling molecules to specific subcellular locations (22, 25).

Analysis of rat NMDA receptor subunit amino acid sequences revealed a proline-rich region in the COOH-terminal portion of the NR2D subunit. This proline-rich region contains sequences that show striking homology to motifs known to bind to SH3 domains (19, 26–28). In the present report, we tested the hypothesis that the proline-rich region of the NR2D subunit interacts with SH3 domains. Our results reveal a selective interaction of the proline-rich region of the NR2D subunit with the SH3 domain of the Abi tyrosine kinase. Co-expression of NR2D with Abi in a model system results in suppression of kinase activity, suggesting a biologically significant interaction between these proteins.

EXPERIMENTAL PROCEDURES

Materials—The anti-Abi monoclonal antibody K-12 and the antiphosphotyrosine antibodies PY20 and PY99 were obtained from Santa Cruz Biotechnology. The anti-Abi antibody 8e9 has been described (29). The M2 anti-FLAG antibody and M2 anti-FLAG antibody resin were obtained from Kodak Scientific Imaging Systems or Sigma. Appropriate
alkaline phosphatase-conjugated secondary antibodies were from Southern Biotechnology Associates. NR1a cDNA was generously provided by Dr. Shigetada Nakanishi (Kyoto University Faculty of Medicine, Kyoto, Japan). NR2D cDNA (6) was the gift of Dr. Peter Seeber (University of Heidelberg, Heidelberg, Germany). GST fusion proteins containing the SH3 domains of Fyn and PLCγ and the full-length Grb2 protein were purchased from Santa Cruz Biotechnology. Glutathione-agarose and protein A-Sepharose were from Sigma.

**Epitope-tagged NR2D Constructs**—The coding sequence of the 8-amino acid FLAG epitope (DYKDDDDK) was added to a COOH-terminal fragment of the NR2D NMDA receptor subunit by PCR to generate the 2D866–1064 construct. This construct contains residues 866–1064 of the rat NR2D subunit, beginning immediately COOH-terminal to the M4 domain at Val^866, extending through Glu^1064, and terminating at the FLAG epitope. The nucleotide sequence of the PCR-amplified fragment was confirmed by automated DNA sequence analysis (Applied Biosystems). 2D^866–1064 was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) for expression in 293T cells.

PCR was used to amplify the COOH-terminal coding region downstream from the unique SacI site. The reverse primer added the FLAG epitope immediately adjacent to the COOH-terminal Val, thereby removing the naturally occurring stop codon. The forward primer included the unique SacI site. The resulting PCR product was subcloned into NR2DpcDNA3 to replace the normal COOH-terminal region. The nucleotide sequence of the PCR-amplified fragment was confirmed by automated sequence analysis (Applied Biosystems).

**Subcloning and Mutagenesis of Abl Constructs**—Abl and ΔSH3 Abl were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The constitutively active Abl linker mutant, Abl-PP (24), was generated by two sequential rounds of mutagenesis in which prolines 242 and 249 were each mutated to alanine using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutants were confirmed by automated DNA sequence analysis (Applied Biosystems).

**Expression of NR2D and Abl in 293T Cells and Preparation of Cell Lysates**—Calcium phosphate-mediated transfection of 293T cells was as described previously (30). 2–3 days after transfection, 293T cells were harvested by centrifugation and lysed. For preparation of total cellular lysates, cells were sonicated in 50 mM Hepes, pH 7.4, 0.5 mM NaCl, 1 mM EDTA, 1 mM MgCl2, 0.1 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM MgCl2, 0.1% Triton X-100 supplemented with 25 μg/ml aprotinin, 50 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and 50 mM Na3MoO4. For immunoprecipitation from cells expressing full-length NR2D, cells were sonicated in 50 mM Hepes, pH 7.4, 0.5 mM NaCl, 1 mM EDTA, 1 mM MgCl2, 5% Triton X-100, 0.1% deoxycholate supplemented with 25 μg/ml aprotinin, 50 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and 50 mM Na3MoO4.

**Immunoprecipitation and Immunoblotting**—For immunoprecipitation, clarified cytosolic extracts were incubated with antibody and protein A-Sepharose for 1 h at 4 °C. Immune complexes were isolated by centrifugation and washed three times with radioimmuno precipitation buffer. Immune complexes were heated in SDS sample buffer. For immunoblotting, cytosolic extracts or immunoprecipitated proteins were run on 4–15% gradient polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated for 1 h with the appropriate primary antibody at 1 μg/ml in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 1.5% bovine serum albumin. The membranes were then washed in TBS-T and probed with a secondary antibody—alkaline phosphatase conjugate in TBS-T containing bovine serum albumin. Following incubation and washing, bound antibody was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech). Membranes were then washed in TBS-T and probed with a secondary antibody—alkaline phosphatase conjugate in TBS-T containing bovine serum albumin. Following incubation and washing, bound antibody was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Expression of GST Fusion Proteins in Escherichia coli**—The coding sequence of the Ab SH3 domain was amplified by PCR using Bcr/Abl as a template and subcloned into the pGEX2T expression vector (Amer sham Pharmacia Biotech). SH3 domains from Src and GAP were subcloned into pGEX2T as described previously (31, 32). The resulting plasmids were used to express the SH3 domains as GST fusion proteins in bacteria. Fusion proteins were purified using glutathione-agarose beads as described previously (31, 33).

**In vitro Association Reactions**—Cytosolic extracts from 293T cells expressing 2D866–1064 were split into aliquots and incubated with the GST-SH3 fusions or GST alone (10 μg/ml) immobilized on glutathione-agarose beads in a final volume of 1.0 ml of lysis buffer. Following incubation at 4 °C for 1 h, protein complexes were pelleted by centrifugation and washed three times with radioimmuno precipitation buffer. Associated proteins were eluted by heating in SDS-PAGE sample buffer, and the presence of 2D866–1064 was determined by immunoblotting with anti-FLAG monoclonal antibody (M2, Santa Cruz Biotechnology).

**RESULTS**

Analysis of deduced rat NMDA receptor subunit amino acid sequences revealed proline-rich sequences in the COOH-terminal portion of the NR2D subunit (Fig. 1). These sequences show striking similarities to those found in SH3 domain binding proteins (27). To determine whether the proline-rich domain of the NR2D subunit can act as an SH3 domain binding protein, in vitro SH3 domain binding assays were performed. A fragment of the NR2D cDNA, encoding amino acids 866–1064, was amplified by PCR and tagged at its COOH terminus with the 8-amino acid FLAG epitope. The resulting construct, 2D^866–1064, was expressed in 293T human embryonic kidney cells and expression was verified by immunoblot and immunoprecipitation using anti-FLAG antibodies (Fig. 2). Lysates from 293T cells expressing 2D^866–1064 were incubated with recombinant immobilized SH3 domain-GST fusion proteins, expressed and purified from E. coli. Following incubation and washing, bound 2D^866–1064 was visualized by immunoblotting with anti-FLAG antibodies. As shown in Fig. 2, the proline-rich fragment of the NR2D subunit bound only to the Ab SH3 domain. 2D^866–1064 did not bind to other SH3 domains tested, including those from phospholipase C-γ, Src, Fyn, GAP, or the full-length Grb2 protein. In addition, 2D^866–1064 did not bind GST alone. Lysates from nontransfected control cells did not bind to any of the SH3 domains tested (data not shown).

The interaction of the Ab SH3 domain with the proline-rich region of NR2D in vitro suggests that these two proteins may associate in a living cell. Co-immunoprecipitation experiments were performed to determine whether full-length Abl and NR2D form a stable complex in co-transfected 293T human embryonic kidney cells. As shown in Fig. 3, both NR2D (tagged with the FLAG epitope at its carboxyl terminus) and 2D^866–1064 co-immunoprecipitated with Abl in 293T cells expressing both proteins. NR2D was not seen in the anti-Ab immunoprecipitates from cells expressing either form of NR2D alone (see Fig. 3, center lanes). These data suggest that the presence of NR2D in the anti-Ab immunoprecipitates results from a specific and stable interaction between the full-length proteins.

To investigate whether NR2D and Abl functionally interact in a model system, 293T cells were transiently transfected with Abl and NR2D cDNAs. Transfection of Abl cDNA in a mammalian overexpression system, such as 293T, results in constitutive Ab kinase activity (34, 35). Lysates were prepared from cells co-expressing wild-type Abl (WT) and either the full-length NR2D subunit or 2D^866–1064, the proline-rich fragment of the NR2D subunit, and immunoblotted with anti-phosphotyrosine antibodies. As shown in Fig. 4, overexpression of WT Abl in this system releases the negative regulation of
tyrosine kinase activity as evidenced by autophosphorylation, in agreement with previous studies (34, 35). Surprisingly, co-expression of either full-length NR2D or 2D866–1064 with WT Abl resulted in inhibition of Abl autokinase activity as indicated on the phosphotyrosine blot. Control blots verify expression of Abl and NR2D proteins. The position of NR2D in the presence of NR1 subunit, 293T cells were co-transfected with NR1, NR2D, and Abl alone and in various combinations. Inhibition of Abl autokinase activity was observed in cells expressing Abl in combination with either NR2D alone or NR2D and NR1 but not in cells expressing Abl in combination with NR1 alone (Fig. 6). It is important to note that functional heteromeric receptors are observed in 293T cells2 co-expressing NR1 and NR2D subunits but not in cells expressing NR2D subunits alone (7), suggesting that in the presence of the NR1 subunit, NR2D is inserted in the proper cellular location. Negative regulation of autokinase activity by the Abl SH3 domain has been reported (37). To determine whether the Abl SH3 domain is critical for the inhibitory effect of NR2D on kinase activity, an Abl mutant lacking the SH3 domain (ΔSH3) was co-expressed with NR2D in 293T cells. 2D866–1064 did not inhibit autophosphorylation of ΔSH3 Abl (Fig. 7A), indicating that inhibition by 2D866–1064 is dependent upon the Abl SH3 domain. In contrast to 2D866–1064, co-expression of the full-length NR2D subunit with ΔSH3 Abl produced partial inhibition of autokinase activity, suggesting the presence of additional sites of interaction between NR2D and Abl. Similar results were also obtained with Abl-PP, an Abl mutant containing two point mutations in the SH2-kinase linker region (Fig. 7B). These mutations have been proposed to release negative regulation by the SH3 domain (24). As seen with ΔSH3, 2D866–1064 did not inhibit Abl-PP autokinase activity (Fig. 7B), suggesting that the proper spatial relationship between 2D866–1064 and the Abl SH3 and kinase domains is required for the inhibitory effect. In contrast, the full-length NR2D subunit partially inhibited Abl-PP kinase activity, similar to that seen for ΔSH3 Abl kinase activity.

**DISCUSSION**

One of the most striking features of the NR2D subunit amino acid sequence is the prevalence of proline residues in the
COOH-terminal region of the molecule, immediately downstream from the M4 domain. This proline-rich region localizes to the cytoplasmic surface of the cell membrane (38), making it a potential target for interactions with regulatory molecules. The data in this report provide evidence of a novel interaction between the NR2D subunit of the NMDA receptor and the Abl tyrosine kinase that may involve the proline-rich region of NR2D and the SH3 domain of Abl.

SH3 domain-dependent interactions of ion channels with nonreceptor tyrosine kinases have been reported in other systems. Src kinase inhibits hKv1.5 potassium channel currents in an SH3 domain-dependent manner (39). Given the structural homology between the potassium channel and the NMDA receptor, it is interesting that the proline-rich domain of NR2D does not interact with the Src SH3 domain (see Fig. 2). However, the preferred Src SH3 domain binding motif (RPLPXXXP (26, 27)) is not present in the NR2D subunit. The Abl SH3 domain shows a preference for the peptide sequence XPhiXXPPPϕXP, where ϕ is any hydrophobic residue (27, 28). This consensus sequence is closely approximated in the NR2D subunit (PKAPPPQP; see Fig. 1).

Data presented here demonstrate that complete inhibition of Abl autophosphorylation by NR2D requires the SH3 domain, which has been implicated in the negative regulation of Abl kinase activity. An inhibitory intramolecular association between the Abl SH3 domain and sequences connecting the SH2 and kinase domains (SH2-kinase linker) has been proposed (24), based on an analogous interaction revealed in the x-ray crystal structures of the inactive form of c-Src and the closely related tyrosine kinase Hck (40, 41). Mutations in the Src, Hck, and Abl SH2-kinase linker domains result in kinase
activation (24, 42, 43), presumably by disrupting the intracellular SH3-linker interaction required for maintenance of the inactive state. Other work has shown that interaction with a ligand that binds exclusively to the SH3 domain of Hck induces kinase activation due to displacement of the linker region from the SH3 domain (44, 45). Because 2D666–1064 inhibits rather than stimulates kinase activity, and inhibition requires the SH3 domain of Abl, we conclude that 2D666–1064 must make additional contacts with Abl outside of the SH3 domain, possibly within the kinase domain itself. Furthermore, the inability of the NR2D proline-rich fragment to inhibit the activated linker mutant of Abl suggests that inhibition requires the wild-type structural relationship between the Abl SH3, linker, and kinase domains. In addition to the NR2D subunit of the NMDA receptor, a number of other proteins have been reported to interact with the Abl SH3 domain. Among these proteins, both AAP-1 (46) and PAG (47) have been shown to suppress kinase activity.

Data presented in this report provide the first demonstration of a direct effect of a ligand gated ion channel subunit on tyrosine kinase function and may have important implications for the significance of Abl in the brain. Although specific functions for the Abl tyrosine kinase have not been determined, Abl plays a role in the negative regulation of cell growth (35) and interacts with cell cycle regulatory proteins (48–50). In addition, recent evidence suggests a role for Abl in mediating the cellular stress response by activating the c-Jun NH2-terminal kinase pathway (51). Interestingly, the c-Jun NH2-terminal kinase pathway is also activated by NMDA receptor stimulation (52, 53), suggesting the possibility that NMDA receptor-mediated activation of cellular stress pathways may be Abl-mediated.

The localization of Abl and NMDA receptor subunits show several parallels. Subcellularly, Abl localizes to both the cytoskeleton and the nucleus (54, 55). The NMDA receptor associates with structural proteins that link it to the cytoskeleton (17, 25). The co-localization of Abl and NMDA receptors to the same subcellular compartment may provide an opportunity for these proteins to interact in a biologically relevant manner. Abl has been identified in the brain, in regions known to express NMDA receptor subunits (56–58), and the same subcellular compartment may provide an opportunity for interaction between NR2D and Abl that may occur during development.

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